BIOASSAY OF MILK FOR ESTROGEN CONTENT FROM STILBESTROL-TREATED AND NON-TREATED COWS

by

ROBERT RICHARD BARON

B. A., Concordia College,
Moorhead, Minnesota, 1952

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Zoology

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1956

TABLE OF CONTENTS

INTRODUCTION AND LITERATURE REVIEW 1

MATERIALS AND METHODS 8

RESULTS 15

DISCUSSION 22

SUMMARY 29

ACKNOWLEDGMENTS 31

LITERATURE CITED 32

INTRODUCTION AND LITERATURE REVIEW

According to Turner (1955), p. 308, diethylstilbestrol, commonly called stilbestrol, is one of the most common artificial estrogens and has the same estrogenic properties as natural estrogens.

Emmens (1950), p. 393, described diethylstilbestrol (4,4' dihydroxy-alpha, beta-diethylstilbene) as a synthetic estrogen which has a potency between that of injected estrone and alpha-estradiol. The three common natural estrogens are alpha-estradiol, estrone, and estriol. Alpha-estradiol is the most active natural estrogen and estriol the least active.

Stilbestrol has been used in many commercial animal feeds for fattening purposes and it has been used to stimulate lactation in virgin heifers. Folley, et al. (1940) showed that copious lactation was produced in goats by the administration of stilbestrol. In a later experiment, Folley, et al. (1941) observed the same results when working with virgin heifers. It is therefore of great importance to determine if the milk so produced contains stilbestrol and, if it does, whether the quantities present could have any effect on the consumers of the milk. As a result, some work has been done to determine the estrogen levels of normal milk and of milk from cattle treated with stilbestrol.

The Department of Dairy Husbandry, Kansas State College, Manhattan, Kansas, after treating dairy cattle with stilbestrol, took milk samples from the treated and also from normal cows and

prepared estrogen extracts of the normal milk and of milk from cattle treated with stilbestrol. These extracts were subsequently used in the present study to determine the estrogen level of normal milk and the estrogen level of milk from cattle treated with stilbestrol.

In an attempt to determine estrogen levels in milk, Lawson, et al. (1945) used a vaginal smear method of bioassay, and tentatively concluded that the concentration of estrogens present, in milk from cattle treated with synthetic estrogens, was between 0.5 and 1.0 micrograms per pint of milk but the concentration is usually much lower. In their studies with milk from normal cows, they obtained no response with doses equivalent to 756 ml of milk in the ovariectomized rat. Two modes of administering the extract were used, namely by subcutaneous injection and intravaginal implantation.

Pope and Roy (1953) also working with milk, described a method of extracting and evaluating the free and the conjugated estrogens. The assays were mainly on bovine colostrum in which estrogenic activity about equal in amount to that found in bovine and human pregnancy blood was detected. The normal milk that was assayed showed no response at a dose level of 20 ml of milk per mouse.

According to Münch (1954) there are estrogens and no androgens in milk. It was found that the estrogen level in milk varied during the heat cycle and that the percentage of estrogen present in milk increased progressively with gestation.

Bioassays of estrogenic material date back to 1923. Allen and Doisy (1923) observed that cyclic responses could be produced in castrate mice by injecting an oily solution of ovarian extracts into the mice. The cyclic responses of the genital tract were identified by microscopic examination of vaginal smears. According to Allen and Doisy (1927) certain other reactions were induced in ovariectomized rats when injected with ovarian hormones. The reactions included a hyperemia and growth in all parts of the reproductive tract. Congestion and swelling of the reproductive tract and other external signs of estrous were also observed.

Using immature albino rats, Wallen-Lawrence (1934) described an assay for the separation of a follicle stimulating hormone and a lutenizing hormone from the anterior lobe of the pituitary body. The evaluation of the potency of a given preparation was based on the weight response of the ovary and the uterus.

Bülbring and Burn (1935) used the spayed rat uteri as a basis of measurement for estrogen assays. Two days after ovariectomy, estrin in oil was injected for four consecutive days. On the eighth day after ovariectomy, the uteri were removed, fixed, and weighed. It was found that the mean increase in the weight of the uterus was proportional to the logarithm of the dose.

Using immature female rats, Dorfman, et al. (1936) performed an assay of theelin, theelol, and estrogenic substances from male human urine. An increase in uterine weight was used as an indication of activity.

After injecting mice with theelin, Allen, et al. (1937) made histological studies of the genital organs and observed that after

a single injection of the estrogen in oil, growth was stimulated prior to nine and one-half hours. Maximal growth activity occurred at 37 hours and declined slightly by 48 hours. The growth activity which was observed was restricted to the epithelial tissues of the genital organs.

Levin and Tyndale (1937) described a quantitative assay for follicle-stimulating substances. The criterion used was the weight response of both the ovary and the uterus. It was concluded that the weight response of the mouse ovary involved a possible error of at least 100 per cent. The uterine weight response was found to be a sensitive, accurate criterion.

According to Astwood (1938) an accurate assay of estrogenic substances could be performed in six hours. It was observed that after a rat received a single injection of 0.1 mg of estradiol, there was a prompt increase in uterine weight, reaching a maximum in six hours, followed by a decrease in weight. The increase in weight was accompanied by and almost entirely due to an increase in the water content of the uterus. Astwood based his method on the increase in water content resulting in an increased uterine weight in immature female albino rats.

According to Lauson, et al. (1939) the assay method based on an increase in uterine weight of immature rats was especially well adapted to the assay of clinical materials, such as blood and urine. The adaptableness of this method to clinical materials was due to the fact that when assaying these materials, the quantity of estrogen is often very limited. Relatively great accuracy was

obtained consistently in the experiment when as few as one to four rats were used in each assay.

Evans, et al. (1940 a,b) performed an assay of gonadotropic substances using the mouse, rat, and chick. The criterion of all assays was the increase in weight of the ovary and the uterus. It was concluded that the mouse was more sensitive and gives a more uniform response in gonadotropic assays. Later, Hauschildt and Evans (1942) assayed chorionic gonadotropins using the increase in uterine weight as a basis of measurement and found that there was no constant dose-response relationship between the gonadotropic hormone of human pregnancy urine and the uterine weight of mice. It was concluded that the mouse-uterine-weight method was unsuitable for the assay of this hormone.

The effects of continuous injections of stilbestrol in adult female rats were described by Morrell and Hart (1941). In a castrate control group it was observed that the uteri were atrophied, small in cross section, and with a slit-like lumen. In a castrate group injected with stilbestrol, the uterus had characteristic structures found in all the various stages of the estrous cycle of the normal untreated rat. It was concluded that there was very little relationship between dosage and the effect on the uterine structure.

Evans, et al. (1941) described a method of estrogen assay using the uterine weight of immature mice as the basis of measurement. It was found that the sensitivity and relative consistency of the method made it a very reliable method of assay for

estrogens. The results indicate that the immature mouse assay is more accurate than the assay using rats as described by Lauson, et al. (1939). However, Evans, et al. (1941) observed that it was necessary to make all assays in conjunction with controls and with several levels of standards.

Emery, et al. (1941) made a study of the duration of estrous following administration of stilbestrol at various sites. Using castrate rats, it was observed that the duration of activity was shortest when the extracts were given intraperitoneally and longest when intramuscular injections were used. Subcutaneous injections caused an activity about midway in duration between the intraperitoneal and intramuscular methods.

Using spayed rats, Kock (1942) performed an assay of pure estrogens and urine extracts. The criterion of activity used was a direct reading of vaginal smears, without staining.

The use of the chick oviduct response, as a basis of measurement, was used by Dorfman and Dorfman (1948). In order to account for body weight variation, all results were expressed as 100 times the ratio of the weight of the oviduct (in milligrams) to body weight in grams. It was concluded that this method was not sensitive, but was precise. In comparing the activity of four estrogens, Dorfman, et al. (1948) found the error range to be -29 to +41 per cent for one of the estrogens assayed. However, the other three were determined with an accuracy of $\pm$10 per cent or better.

Rubin, et al. (1951) employed the uterine weight method of

assay for assaying the natural estrogens. The method was based on the straight line relationship between the logarithm of the dose and the uterine weight response of immature female mice. The mice were injected subcutaneously once daily for three days and sacrificed 24 hours after the last injection. All of the results were expressed as uterine ratios.

Dorfman and Dorfman (1953) presented a quantitative method for the assay of estrogens by oral administration to the chick. The increase in uterine weight was used as an indication of activity and it was stated that the method was convenient, inexpensive, and that the results were reproducible. Later, Dorfman and Dorfman (1954) described a similar method of assay using the uterine weight of immature rats, after receiving injections of estrogens, as an indication of activity. All of the results were expressed as uterine ratios.

Stob, et al. (1954) described a technique for the detection of estrogen in meat of hormone treated animals. Under the conditions of the method used, the uterine weight response assay was four times more sensitive for the detection of orally administered diethylstilbestrol than the vaginal smear method. It was shown that there was residual hormone activity in beef secured from steers 168 days after treatment with synthetic estrogens and progesterone.

MATERIALS AND METHODS

Before adopting the standard assay procedure, which will be described later, a number of preliminary experiments were performed. The preliminary experiments were as follows.

In experiment one, seven day old female Leghorn chicks were used in an assay of powdered milk from cattle treated with stilbestrol. An increase in uterine weight was used as the basis of measurement for stimulation. The powdered milk was dissolved in water (0.74 gm milk per ml of water) and was administered by means of a pipette directly into the crop of the chick. Four groups, with seven chicks in each group, were used in this experiment. Each chick in group one received an equivalent of 29.6 gm of powdered milk from a cow treated with stilbestrol and group two was a control group. Group three received an equivalent of 52.5 gm of milk, per chick, from a cow treated with stilbestrol and group four was a control group. The chicks were sacrificed three days after the last treatment and the uteri were removed from the body cavity and weighed.

The second experiment was performed in the same manner as experiment one. However, condensed milk from stilbestrol treated cows was used instead of powdered milk. Five chicks were used in each of two groups; one group was treated with the condensed milk from a stilbestrol treated cow and the other group was used as a control group. The treated group received 180 ml of condensed milk, per chick, over a nine day period. Three days after the last treatment, the chicks were sacrificed and the uteri were

taken from the body cavity and weighed.

The third study in this group of preliminary experiments was the first experiment in which ovariectomized mice were used. An increase in uterine weight was used as the criterion of measurement for stimulation. Three groups of mice were used; one treated group of eight mice, a second treated group of five mice, and a control group of five mice. The treated groups were fed powdered milk for 14 days. Group one was fed powdered milk prepared from milk taken from a cow treated with stilbestrol and each mouse consumed approximately 27 gm of the powdered milk. The second treated group was fed powdered milk prepared from the milk of a non-treated cow and each mouse consumed approximately 29 gm of powdered milk. The third group, the controls, was fed a routine diet without powdered milk. On the fourteenth day, the mice were killed and the uteri were removed from the body cavity and weighed.

The fifth experiment was the first study in which estrogen extracts, prepared from milk and dissolved in olive oil, were used. The extract, in olive oil, was injected into the mice subcutaneously and the daily dose was 0.1 cc of extract and oil per mouse. Seven groups were used in the experiment with four mice in each group. Groups one, two, and three received an extract prepared from whole milk (475 ml) taken from a cow which had been treated with stilbestrol. The total extract was dissolved in 10 cc of olive oil. Each mouse in group one received a total dosage of 0.3 cc of extract and oil, equivalent to 14.25 ml of milk per mouse. Group two received 0.6 cc, per mouse, of the

same extract or a milk equivalent of 28.5 ml of milk per mouse. Each mouse in group three received 1.0 cc of extract and olive oil solution, equivalent to 47.5 ml of milk per mouse. Groups four, five, and six were treated with an extract prepared from 2000 ml of condensed milk taken from a cow which had been treated with stilbestrol. Group four received 0.3 cc, per mouse, of extract and oil, which was equivalent to 60 ml of milk per mouse. The total dose which group five received was 0.6 cc of extract and oil, per mouse, and this was equivalent to 120 ml of milk per mouse. Group six received 1.0 cc of extract and oil, per mouse, or a milk equivalent of 200 ml of milk per mouse. The seventh group of mice, which was not treated, was used as the control group. Twenty-four hours after the last injection, the mice were killed, the individual body weights were determined, and the uteri were removed from the body cavity and weighed.

Experiment six was an assay performed with four levels of stilbestrol standards. The standards were prepared by dissolving a known quantity of stilbestrol in 10 cc of olive oil. Five groups of mice with seven mice in each group were used in this experiment. The dosage levels of the five groups were as follows: group one received 0.025 mg of stilbestrol, per mouse, in 0.4 cc of olive oil; group two received 0.05 mg of stilbestrol, per mouse, in 0.4 cc of olive oil; group three received 0.1 mg of stilbestrol, per mouse, in 0.4 cc of olive oil; group four received 0.2 mg of stilbestrol, per mouse, in 0.4 cc of olive oil; and group five, which was the control group, received 0.4 cc of

olive oil. Each mouse was injected once daily for four days with 0.1 cc of stilbestrol and olive oil. Twenty-four hours after the last injection, the mice were sacrificed. The individual body weights were determined and the uteri were taken from the body cavity and weighed.

The method used by the Department of Dairy Husbandry in preparing the estrogen extracts from milk was that described by Pope and Roy (1953). In the extraction of the free estrogens, whole milk (100 ml) was refluxed for ten minutes with methanol (1000 ml), cooled to 30 degrees, and filtered. The residue was again refluxed with methanol (400 ml) for 30 minutes. The combined filtrates were evaporated to dryness at reduced pressure, and saturated aqueous $NaHCO_3$ solution (200 ml) and ether (100 ml) added. After separation, the $NaHCO_3$ phase was washed with more ether (6 x 200 ml) and the combined ether solution, after drying over Na_2SO_4, evaporated to dryness. The residue was dissolved in a mixture of CCl_4 (180 ml) and ether (5 ml), and this solution extracted with portions of aqueous N-KOH (200, 100, 100 ml). The combined alkaline solution was then brought to pH 1 with concentrated HCl and extracted with ether (5 x 200 ml). The ether solution was washed with saturated aqueous $NaHCO_3$ (2 x 20 ml), dried over Na_2SO_4 and evaporated to dryness. The process was then repeated on a second portion (100 ml) of milk and the two final products combined.

For the extraction of the conjugated estrogens, the combined $NaHCO_3$ solutions from the process described above were brought to

pH 1 with concentrated HCl, mixed with concentrated HCl (40 ml), and boiled for five minutes. The cooled solution was then made slightly alkaline, brought again to pH 1, and extracted with ether (5 x 200 ml). The ether solution was washed with several 20 ml portions of water until these were neutral, dried over Na_2SO_4, and evaporated to dryness.

The free estrogen extract and the conjugated estrogen extract were then combined and the total extract was dissolved in olive oil.

The milk extracts, which were used in this study, were prepared from milk samples taken from the dairy cattle described in Table 1.

Table 1. Data on cows from which milk samples were taken.

Test group	Breed	Dosage level (mg/1000 lb. bd. wt./day)	Duration of treatment (days)	Age of cow (years)	Duration of pregnancy (days)	Duration of lactation (months)
1	Jersey	10	116	2	90	5½
2	Jersey	0	0	2	Not pregnant	5½
3	Jersey	20	13	2	Not pregnant	5½
4	Holstein	0	0	5	Not pregnant	6
5	Holstein	10	124	5	55	6
6	Holstein	20	125	4	120	7
7	Brown Swiss	0	0	3	60	5
8	Brown Swiss	0	0	3	60	5

bd. wt. = body weight.

The method employed in this study for estrogen assay was essentially the method described by Evans, et al. (1941) with modifications including the use of ovariectomized mice, injected once

daily with 0.1 cc oil solution of extract for four days. Seven animals were used on each level and 48 hours after the last injection the mice were killed and the uteri weighed. The results were expressed as uterine ratios.

The female albino mice used in this study were obtained from Fountain's Small Stock Farm, Ashland, Wisconsin. In this laboratory the mice were fed Purina Laboratory Chow which was assayed by the Ralston Purina Co., St. Louis, Missouri, for estrogenic activity and found to be essentially estrogen free. The mice were approximately six weeks old when received and were bilaterally ovariectomized when they were eight weeks old.

The operative surgery during ovariectomy was as follows: Ether was used as the anesthetic and was placed in a test tube with cotton in the bottom and the test tube was placed over the head of the mouse. Extreme care was needed to keep the mouse in a light state of anesthesia during the operation as a slight excess would be fatal. After the mouse was in a state of anesthesia, the hair was trimmed from the general region where the incisions were to be made. A transverse incision was made in the skin of the lumbar region on the right side. The skin was shifted so the incision was over the right ovary, which was visible through the abdominal wall embedded in a small piece of perovarian fat. A small incision was made through the dorsal musculature to admit the tip of a pair of fine forceps. The fat was grasped with a pair of forceps and pulled out through the incision in the body wall. With another pair of forceps, the tip

of the uterine horn was held and the ovary, intact in its capsule, together with the Fallopian tube, was excised. The horn of the uterus was released and allowed to slip back into the abdominal cavity. After the right ovary had been removed, the same procedure was followed in removing the left ovary.

Usually there was no bleeding and it was not necessary to suture the abdominal wall. Aseptic conditions were not necessary; however, a small amount of sulfadiazine was placed in the incision to prevent infection.

One week after the mice were ovariectomized, the assay was started. Three experiments were run, two of which consisted of two milk extracts, and the third with three milk extracts. One milk extract was prepared by adding a known quantity of stilbestrol to 100 cc of normal milk, and the estrogens were extracted from the milk by the normal procedure. In each experiment three standard groups and one control group were run in conjunction with the milk extract groups. Seven mice were used in each group, making a total of 133 mice used: seven milk extract groups, nine standard groups, and three control groups.

The mice were injected subcutaneously once daily with the appropriate dose. The daily dose of the milk extracts, which were dissolved in olive oil, was 0.1 cc of extract and oil and the total dosage was 0.4 cc during a four day injection period. The three standard groups were injected with different concentrations of stilbestrol. The stilbestrol was dissolved in olive oil and the daily dose of steroid was contained in 0.1 cc of olive oil

and the total dosage over the four day injection period was contained in 0.4 cc of olive oil. The powdered stilbestrol used in preparing the standards was obtained from the Research Chemicals Division of George A. Breon and Co., Inc., Kansas City, Missouri. The control group was injected with 0.1 cc of olive oil each day during the four day injection period and the total dosage was 0.4 cc of olive oil.

The mice were sacrificed 48 hours after the last injection and the individual body weights were determined. The uterus was separated from the vagina by cutting through the cervix; the surrounding tissue was stripped off and the uterus removed from the body cavity. After pressing out the intra-uterine fluid on blotting paper, the uterus was weighed on a Fisher Gram-atic Balance to the nearest 0.1 mg. After the uterus had been weighed it was fixed in Bouin's Fluid for storage and possible histological studies.

The criterion used to detect estrogenic activity was an increase in uterine weight. All the results were expressed as uterine ratios, which are defined as the weight of the uterus (in milligrams) per gram of body weight times 100.

RESULTS

The first preliminary experiment which was performed, using the chick oviduct as the basis of measurement for stimulation, showed no response to the treatment. The average uterine weight of group one, which received an equivalent of 29.6 gm of powdered milk per chick, was 22.1 mg. The second group, which was the

control group, had an average uterine weight of 20.34 mg. Group three received an equivalent of 52.50 gm of powdered milk per chick and the average uterine weight was 34.5 mg. The control group for group three (group four) had an average uterine weight of 35.8 mg.

In the second experiment, condensed milk was used instead of powdered milk. The increase in uterine weight of the treated group over the control group was not enough to be considered a positive response. The average uterine weight of the treated group, which received 180 ml of condensed milk per chick, was 73.8 mg and the average uterine weight of the control group was 66.6 mg.

Ovariectomized mice were employed in the third experiment of the preliminary studies and an increase in uterine weight was used as the criterion for measurement for stimulation. The mice in group one, which received approximately 27 gm of powdered milk per mouse, from a stilbestrol treated cow, had an average uterine weight of 22.05 mg. The second group of mice was fed approximately 29 gm of powdered milk per mouse, and the average uterine weight was 21.78 mg. The control group, which was fed a routine diet, had an average uterine weight of 23.9 mg. The uterine weights of the three groups, two treated and one control, indicated that there was no response in either of the treated groups.

The fourth experiment was conducted in the same manner as described for experiment three. Group one was fed powdered milk

(approximately 25 gm/mouse) from a non-treated cow and the average uterine weight for the group was 23.38 mg. The second group was fed powdered milk from a non-treated cow and each mouse consumed approximately 31 gm of powdered milk. The average uterine weight of this group was 21.12 mg. The control group had an average uterine weight of 27.14 mg. The results of this experiment, expressed as uterine weights, indicated that there was no response to the treatment in either of the treated groups.

In experiment five, estrogen extracts prepared from milk of cows which had been treated with stilbestrol were assayed. Three groups were treated with an extract prepared from 475 ml of whole milk. Group one received an equivalent of 14.25 ml of milk per mouse and the average uterine weight was 9.1 mg. The second group received a milk equivalent of 28.5 ml of milk per mouse and the average uterine weight was 7.67 mg. Group three was treated with a milk equivalent of 47.5 ml of milk per mouse and the average uterine weight was 8.97 mg. Groups four, five, and six were treated with an extract prepared from 2000 ml of condensed milk taken from a cow which had been treated with stilbestrol. Group four was treated with a dosage equivalent to 60 ml of milk per mouse and the average uterine weight was 9.82 mg. The fifth group received a milk equivalent of 120 ml of milk per mouse and the average uterine weight was 10.93 mg. The last treated group, group six, received a milk equivalent of 200 ml of milk per mouse and the average uterine weight was 11.67 mg. Group seven, which was the control group, was not treated and the average uterine weight was 8.48 mg. The results

of this experiment, expressed as uterine weights, indicated that there was no positive response to any of the various levels of treatment.

A qualitative determination of uterine response to estrogenic stimulation can be made visibly by noting hyperemia of the uterine blood vessels. No such response was noted in any of the experiments mentioned above.

Experiment six, the last experiment of the preliminary studies, was conducted with four levels of stilbestrol standards. The uterine weight of the first group, which received 0.025 mg of stilbestrol per mouse, was 78.87 mg. Group two was treated with 0.05 mg of stilbestrol per mouse and the average uterine weight was 77.14 mg. The mice in group three were treated with 0.1 mg of stilbestrol and the average uterine weight was 79.31 mg. The last treated group received 0.2 mg of stilbestrol per mouse and the average uterine weight was 68.52 mg. The average uterine weight of the control group was 19.37 mg. The results, expressed as average uterine weights, indicated there was stimulation in all treated groups; however, there was no separation in response between the various levels of dosage. This indicated that each dose level caused a maximum response.

The data from the three definitive experiments are shown in Table 2. In each of the experiments a significant difference at the 99.9 per cent probability level (p 1) between the group means was demonstrated.

The standards in each experiment consisted of three dose levels: 3.12 micrograms, 0.39 micrograms, and 0.048 micrograms

Table 2. Data from the three definitive experiments.

Test group	Volume of milk extracted (ml)	Volume of extract and olive oil (cc)	Stilbestrol added ug/0.1 cc	Total dose (cc)	No. of mice	Milk equivalent per mouse (ml)	Average body weight (gm)	Average uterine weight (mg)	Average ratio (mg/gr x 100)
				Experiment I					
1	100	3	---	0.4	7	13.33	21.71	13.62	64.52
2	100	3	---	0.4	7	13.33	22.11	13.90	63.16
St. I	---	-	0.78	0.4	7	---	22.11	62.90	298.48
St. II	---	-	0.097	0.4	7	---	21.40	45.32	212.71
St. III	---	-	0.012	0.4	7	---	18.54	22.61	122.31
Control	---	-	---	0.4	7	---	19.84	13.18	67.45
				Experiment II					
3	100	3	---	0.4	7	13.33	21.28	19.30	92.50
4	100	3	---	0.4	7	13.33	22.60	11.42	51.89
5	100	3	---	0.4	7	13.33	24.18	10.77	45.19
St. I	---	-	0.78	0.4	7	---	19.47	54.52	282.69
St. II	---	-	0.097	0.4	7	---	23.95	49.84	208.80
St. III	---	-	0.012	0.4	7	---	22.57	15.84	71.67
Control	---	-	---	0.4	7	---	22.22	9.81	45.93
				Experiment III					
6	100	5	---	0.4	1	80.00	18.8	10.8	57.44
7	100 + 21.84 ug stil.	3	---	0.4	7	13.33	23.51	31.97	136.64
8	100	3	---	0.4	7	13.33	24.51	13.44	54.91
St. I	---	-	0.78	0.4	7	---	20.72	43.50	210.49
St. II	---	-	0.097	0.4	7	---	19.68	35.44	182.29
St. III	---	-	0.012	0.4	6	---	24.21	17.83	74.80
Control	---	-	---	0.4	7	---	22.18	10.31	46.39

St. = Standard; stil = stilbestrol; ug = microgram.

of stilbestrol. A significant difference (95% p 1) between the means of the standards of each experiment was shown. Moreover, a significant (99.9% p 1) linear response of uterine weight/body weight to the log standard doses of stilbestrol was shown also in the three experiments. A quadratic response was noted in experiments two and three but the linear response was much greater.

In experiment one, group one was treated with an estrogen extract prepared from the milk of a stilbestrol treated cow while group two was treated with an estrogen extract from a non-treated cow. There was no significant difference between the uterine ratios of these groups or between these and the control group. The mean difference between the lowest level standard group (0.048 micrograms stilbestrol) and the control group was 54.86 which is considerably greater than the least significant difference (95% p 1) for these groups which was 21.9.

In experiment two, no significant difference was noted between the lowest level standard group and the control group although the difference, 25.74, approaches the least significant difference (95% p 1) of 27.9 for these groups.

Groups three and five were both treated with extracts from cows which had been treated with stilbestrol. The milk sample for group three was from a cow on high level (20 mg stilbestrol/1000 lb body weight/day for 31 days) and group five from a low level (10 mg stilbestrol/1000 lb body weight/day for 124 days) fed cow. The high level stilbestrol extract group (group three) was significantly different (95% p 1) from the low level group (group five) but not from the lowest level standard group

(0.048 micrograms stilbestrol). Group five was not significantly different from a group (group four) which was treated with an estrogen extract prepared from the milk of a non-treated cow. This is the only case in which an estrogen milk extract (to which stilbestrol was not added) showed a response comparable to the response elicited by a standard dosage of stilbestrol. There was no significant difference between groups four and five and the control group, however, there was a significant difference (95% p 1) between group three and the control group.

In experiment three, group seven was treated with an estrogen extract prepared from the milk of a non-treated cow. Before the extraction was made, a known quantity (21.84 micrograms) of stilbestrol was added to the milk sample. Group eight was treated with an estrogen extract prepared from the milk of the same cow; however, there was no stilbestrol added to the milk sample. The extract group (group seven), which had the stilbestrol added, was significantly different (95% p 1) from group eight which received the same milk sample without stilbestrol added and was also significantly different from the control group. There was no significant difference between group eight and the control group.

In conjunction with experiment three, one mouse (group six) was treated with an estrogen extract prepared from the milk of a cow which had been treated with stilbestrol. The mouse received a milk equivalent of 80 ml and the uterine weight of this mouse was 10.8 mg as compared to 10.31 mg which was the average uterine

weight of the control group. The ratio for group six was 57.44 as compared to 46.39 for the control group. There was some indication of stimulation as witnessed by a slight hyperemia of the uterine blood vessels.

The statistical computations were performed by the Statistics Laboratory of Kansas State College, Manhattan, Kansas.

DISCUSSION

The preliminary studies that were described in this study were performed to find a method of administering milk to experimental animals for an assay of estrogen content. There was no response to the treatment in the first two experiments using chicks; therefore, it was concluded that if there were estrogens present in the milk, they were not concentrated enough to cause a stimulation. In experiment two there was a slight increase in weight of the uteri of the treated group over the control group. However, there was no visible response as witnessed by the absence of hyperemia of the uterine blood vessels and it was concluded that this increase in average uterine weight was not due to estrogen stimulation. The method of administering the milk in this experiment was not satisfactory for quantitative purposes. The measurement of the milk was accurate; however, the quantity that the chick retained in the crop was questionable.

In the experiments in which powdered milk was fed to mice, there was no accurate way to measure the amount of milk consumed as a considerable amount of the material was wasted by the mice.

It was found that this method was time consuming and over a 14 day period approximately 30 gm per mouse was consumed. Even if there was an accurate way to measure the quantity of milk consumed, the method would be impracticable from the standpoint of the time required for the mice to consume an appreciable amount of the powdered milk. There was no visible response of the uteri in the two experiments in which powdered milk was fed to the mice. It was concluded that, if there were estrogens present in the milk, the mice were not consuming a sufficient quantity to cause stimulation.

The last preliminary study that was performed, using milk, employed the use of estrogen extracts from milk. It was found that this was the most satisfactory way of administering the milk to the mice as it was possible to measure accurately the quantity which was given to each mouse. There was no visible response of the uterine blood vessels in this experiment; therefore, it was necessary to run a series of standards (known concentrations of stilbestrol) to determine what concentration of stilbestrol would cause stimulation of the uteri in ovariectomized mice.

In the last experiment, an assay was performed on a series of five stilbestrol standards of known concentration. It was found in this experiment that 0.025 mg of stilbestrol would cause a response of the uterus; however, this response appeared to be a maximum response as there was little difference between the average uterine weight of this group and of groups receiving higher doses. There was a wide separation between the uterine weights

of the control group and the treated groups; therefore, it was concluded that a dose of 0.025 mg of stilbestrol caused a maximum response of the uteri in ovariectomized mice.

The relationship between the stilbestrol standard levels and the uterine ratios of the three experiments in this study are shown in Fig. 1. The standards in each experiment consisted of these dose levels: 3.12 micrograms, 0.39 micrograms, and 0.048 micrograms of stilbestrol. Within each experiment, there was a significant difference (95% p l) between the lowest level standard and the control group. In experiment two the difference between the lowest level standard and the control group was not significant; however, the difference, 25.74, approaches the least significant difference (95% p l) of 27.9 for these groups. The results indicate that in each experiment the low level standard was near the minimal concentration of stilbestrol required to cause stimulation.

In each experiment, there was a sharp increase in the uterine ratio of the medium level standard group over the uterine ratio of the low level standard group. The increase in uterine ratio of the high level stilbestrol standard group over the medium level standard group was not as great in any of the three experiments, being the least in experiment three. These results indicate that the concentration of the high level standard was approaching the concentration of stilbestrol which would cause a maximum response.

It was concluded that when stilbestrol is administered under the stated experimental conditions and at these various levels,

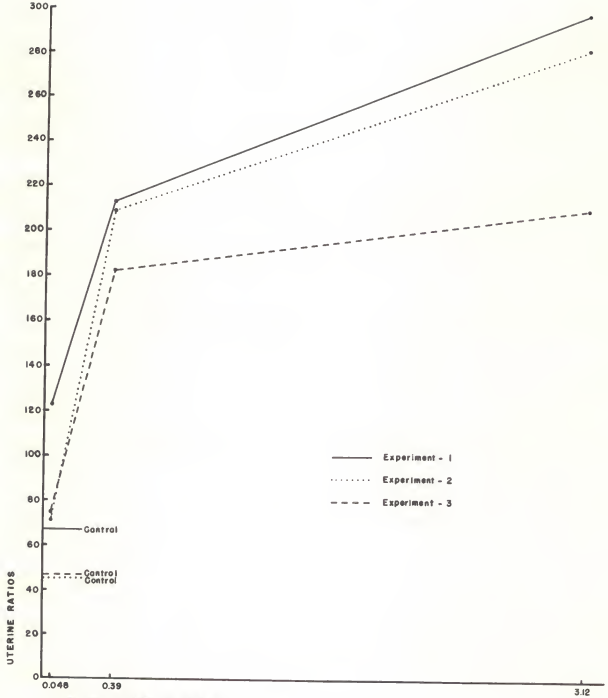

FIG. I. Relationship between stilbestrol standard levels and uterine ratios

the lowest level will cause a minimal response, the amount of response increasing as a linear function of the logarithm of the dose.

In experiment one, two estrogen extracts prepared from milk samples, were assayed. The extract which was administered to group one was prepared from the milk of a cow which had been treated with stilbestrol. Group two was treated with an estrogen extract prepared from the milk of a non-treated cow. There was no significant difference between these groups or between these groups and the control group. The low level standard which caused a significant (95% p 1) response contained 0.048 micrograms of stilbestrol. Each mouse in the two treated groups received a milk equivalent of 13.33 ml of milk. The results indicated that if estrogens were present in the milk samples, they were present in concentrations less than 0.048 micrograms per 13.33 ml of milk.

Groups three and five, experiment two, were treated with the extracts prepared from the milk of stilbestrol treated cows and group four of the same experiment, was treated with the extract prepared from the milk of a non-treated cow. There was no significant difference between groups four and five or between these groups and the control group. The low level standard (0.048 micrograms stilbestrol) was not significantly different from the control group; however, the difference, 25.74, approaches the least significant difference (95% p 1) of 27.9 for these groups. Each mouse received an equivalent of 13.33 ml of milk and the results indicated if there were estrogens present in the milk

samples used in groups four and five, it was less than 0.048 micrograms per 13.33 ml of milk. The difference in response of group three and the control group was significant at the 95 per cent probability level. The uterine ratio of the low level standard was 71.67 and the ratio of group three was 92.50; these results indicated that there was slightly more than 0.048 micrograms of estrogen per 13.33 ml of milk.

In experiment three, two estrogen extracts were assayed. The milk samples from which the extracts were prepared for groups seven and eight were from the same cow; however, before the sample for group seven was extracted, 21.84 micrograms of stilbestrol were added. There was no significant difference between the response of group eight and the control group; however, the difference in response between group seven and the control group was significant at the 95 per cent level. The total dosage of standard one was 3.12 micrograms of stilbestrol. Assuming that this level caused a maximum response and assuming 100 per cent recovery of the stilbestrol in the extraction process, the total dosage of group seven was 2.91 micrograms of stilbestrol. However, there was a difference in the response of standard group one and group seven; therefore, by comparing the uterine ratios of these two groups, it was concluded that approximately 70 per cent of the stilbestrol was recovered in the extraction process.

One mouse, group six, which received a milk equivalent of 80 ml from a cow treated with stilbestrol, showed a slight indication of stimulation. Since only one mouse was used as the group,

this datum was not statistically analyzed and no conclusion was made.

The standard stilbestrol level tests show that it is possible to measure estrogenic activity by the mouse uterine weight method. In all of the standards, there was a response of the uteri to the stilbestrol stimulation; however, in the groups treated with estrogen extracts prepared from milk, there was a significant (95% p 1) response in only one group. Therefore, if there are estrogens present in the milk, their level after extraction is usually too low to cause a stimulation of the uteri of mice where each mouse received a quantity of extract equivalent to 13.33 ml of milk. It can be concluded that in the estrogen extracts prepared from milk samples where no response was observed, there was less than 0.048 micrograms of estrogen per 13.33 ml of milk.

In the group in which a response was observed, it was hard to predict or assign a specific value to the amount of estrogen present because of the variation between mice and also because the increase in response from the low level standard to the medium level standard was so steep. Therefore; it can be concluded that this method is a good qualitative method for determining the presence of estrogen but not an accurate quantitative test. The method, as described in this paper, can profitably be used by future workers for a qualitative determination of estrogens.

The method of assay used in this experiment was found to be convenient and a minimum amount of time was required for the

actual assay. The use of immature mice, if available, would increase the convenience as the ovariectomization of the mice was found to be the most time consuming part of the assay.

There are several factors which should be considered in future work with this method. First of all, it would be advisable to use larger numbers of mice in each group in an attempt to decrease the variance between groups. Secondly, the estrogen extracts should be prepared from larger quantities of milk so that the milk equivalent per mouse would be considerably greater than it was in this study. The third factor would be to use immature mice and the last important consideration would be to assay more estrogen extracts prepared from milk samples to which known quantities of stilbestrol had been added. This would aid in establishing the efficiency of the extraction process and estimating the percentage recovery of the estrogens present in the milk.

SUMMARY

1. A total of 133 ovariectomized mice were subcutaneously injected with stilbestrol, estrogen extracts prepared from the milk of cows treated with stilbestrol and from non-treated cows, and with olive oil.

2. The standards in each experiment consisted of three dose levels: 3.12 micrograms, 0.39 micrograms, and 0.048 micrograms of stilbestrol. A significant difference (95% p l) between the means of the standard groups of each experiment was shown.

3. One group treated with an estrogen extract prepared from the milk of a cow treated with stilbestrol, showed a positive response.

4. The method as described is considered to be a qualitative determination for estrogens.

5. Six groups: three treated with estrogen extracts, prepared from milk of cows treated with stilbestrol, and three treated with estrogen extracts, prepared from the milk of non-treated cows, showed no response to the treatment.

6. One group which was treated with an estrogen extract prepared from the milk of a normal cow, to which a known quantity of stilbestrol was added, showed a significant response.

ACKNOWLEDGMENTS

The author wishes gratefully to acknowledge Dr. E. H. Herrick for his advice and criticism during this study, and for his assistance given during the assay procedure; and the George A. Breon and Co., Inc., for supplying the diethylstilbestrol.

LITERATURE CITED

Allen, Edgar, and Edward A. Doisy.
An ovarian hormone, preliminary report on its localization, extraction, and partial purification, and action in test animals. Jour. Amer. Med. Assoc. 81:819-821. 1923.

Allen, Edgar, and Edward A. Doisy.
Ovarian and placental hormones. Physiol. Rev. 7:600-650. 1927.

Allen, Edgar, G. M. Smith, and W. U. Gardner.
Accentuation of the growth effect of theelin on genital tissues of the ovariectomized mouse by arrest of mitosis with colchicine. Amer. Jour. Anat. 61:321-341. 1937.

Astwood, E. B.
A six-hour assay for the quantitative determination of estrogen. Endocrinol. 23:25-31. 1938.

Bülbring, Edith, and J. H. Burn.
The estimation of oestrin and of male hormone in oily solutions. Jour. Physiol. 85:320-333. 1935.

Dorfman, Ralph I., and Adeline S. Dorfman.
Studies on the bioassay of hormones - the assay of estrogen by a chick oviduct method. Endocrinol. 42:85-92. 1948.

Dorfman, Ralph I., and Adeline S. Dorfman.
The assay of estrogens in the chick by oral administration. Endocrinol. 53:301-305. 1953.

Dorfman, Ralph I., and Adeline S. Dorfman.
Estrogen assays using the rat uterus. Endocrinol. 55:65-69. 1954.

Dorfman, Ralph I., Adeline S. Dorfman, and Dorotha Grim.
Studies on the bioassays of hormones - comparative activity of estrogens by the oviduct response in chicks. Endocrinol. 42:98-101. 1948.

Dorfman, Ralph I., T. F. Gallagher, and F. C. Koch.
The nature of the estrogenic substance in human male urine and bull testis. Endocrinol. 19:33-41. 1936.

Emery, Frederick E., Charles S. Matthews, and Edward L. Schwabe.
Duration of estrus following intraperitoneal, oral, intrathoracic, subcutaneous, and intramuscular administration of stilbestrol. Endocrinol. 29:1028-1030. 1941.

Emmens, C. W.
Hormone assay. New York: Academic Press Inc., 1950. 556 p.

Evans, John S., Leonard Hines, Joseph J. Ceithaml, and F. C. Koch. Comparative study on the augmentation of the action of gonadotropins from various sources in the rat, mouse, and chick. Endocrinol. 26:1012-1021. 1940a.

Evans, John S., Leonard Hines, Roger Varney, and F. C. Kock. The comparative assay of gonadotropic substances on rats, mice, and chicks. Endocrinol. 26:1005-1011. 1940b.

Evans, John S., Roger F. Varney, and F. C. Kock.
The mouse-uterine weight method for the assay of estrogens. Endocrinol. 28:747-752. 1941.

Folley, S. J., Helen M. Scott Watson, and A. C. Bottomley.
Induction of lactation in goats with diethylstilbestrol dipropionate. Jour. Physiol. 98:15p. 1940.

Folley, S. J., Helen M. Scott Watson, and A. C. Bottomley.
Initiation of lactation in nulliparous heifers by diethylstilbestrol. Jour. Physiol. 100:7p. 1941.

Hauschildt, James D., and John S. Evans.
Unsuitability of the mouse-uterine-weight method for assay of chorionic gonadotropin. Endocrinol. 30:392-394. 1942.

Kock, F. C.
A spayed-rat estrogen assay method and its application to pure estrogens and urine extracts. Endocrinol. 31:162-166. 1942.

Lauson, H. D., Carl G. Heller, June B. Golden, and E. L. Sevringhaus. The immature rat uterus in the assay of estrogenic substances, and a comparison of estradiol, estrone and estriol. Endocrinol. 24:35-44. 1939.

Lawson, W., S. W. Stroud, and P. C. Williams.
Oestrogen excretion in milk from oestrogenized cattle. Jour. Endocrinol. 4:83-89. 1945.

Levin, Louis, and H. H. Tyndale.
The quantitative assay of follicle stimulating substances. Endocrinol. 21:619-628. 1937.

Morrell, J. A., and G. W. Hart.
Studies on stilbestrol I. Some effects of continuous injections of stilbestrol in the adult female rat. Endocrinol. 29:796-808. 1941.

Münch, Udo.
Die ausscheidung von natürlichen androgenen und östrogenen in der milch. Milchwissenschaft. 9:150-153. 1954.

Pope, G. S., and J. H. B. Roy.
The oestrogenic activity of bovine colostrum. Biochem. Jour. 53:427-430. 1953.

Rubin, Betty L., Adeline S. Dorfman, Lila Black, and Ralph I. Dorfman. Bioassays of estrogens using the mouse uterine response. Endocrinol. 49:429-439. 1951.

Stob, Martin, F. N. Andrews, and M. X. Zarrow.
Detection of residual hormone in meat of animals treated with synthetic estrogens. Amer. Jour. Veter. Res. 15:319-322. 1954.

Turner, C. Donnell.
General endocrinology. 2nd ed. Philadelphia and London: W. B. Saunders, 1955. 553 p.

Wallen-Lawrence, Z.
Proof of the existence of a follicle-stimulating and a lutenizing hormone in the anterior lobe of the pituitary body. Jour. Pharmacol. and Exptl. Therap. 51:263-286. 1934.

BIOASSAY OF MILK FOR ESTROGEN CONTENT FROM STILBESTROL-TREATED AND NON-TREATED COWS

by

ROBERT RICHARD BARON

B. A., Concordia College,
Moorhead, Minnesota, 1952

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Zoology

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1956

Stilbestrol has been used in many commercial animal feeds for fattening purposes and to stimulate lactation in virgin heifers. Therefore, some work has been done to determine if the milk so produced contains stilbestrol and, if it does, whether the quantities present could have any effect on the consumers of the milk. The purpose of this study was to determine the estrogen levels of milk samples from cows which had been treated with stilbestrol and from non-treated cows.

Estrogen extracts were prepared from milk samples (100 ml), dissolved in olive oil (3 cc), and administered to ovariectomized mice. The mice were injected subcutaneously once daily with 0.1 cc of the extract and olive oil. The total dosage over the four day injection period was 0.4 cc, which was equivalent to 13.33 ml of milk per mouse. Three experiments were run, two of which consisted of two milk extracts, and the third with three milk extracts. One of the milk extracts was prepared by adding a known quantity of stilbestrol (21.84 micrograms) to 100 cc of milk from a non-treated cow and extracting the estrogens from the milk. In each experiment, three standard groups and one control group were run in conjunction with the milk extracts. The standard groups in each experiment were injected with 0.1 cc of oil and stilbestrol each day for four days and the total dosages were 3.12 micrograms, 0.39 micrograms, and 0.048 micrograms of stilbestrol per mouse. Seven mice were used in each group, making a total of 133 mice; seven milk extract groups (four groups received estrogen extracts prepared from the milk of stilbestrol

treated cows and four groups received extracts of the milk from non-treated cows), nine standard groups, and three control groups.

The criterion used to detect estrogenic activity was an increase in uterine weight. All the results were expressed as uterine ratios, which are defined as the weight of the uterus (in milligrams) per gram of body weight times 100.

A significant difference at the 95 per cent probability level was shown between the means of the standard groups of each experiment. One group, which was treated with an estrogen extract, prepared from the milk of a cow which had been treated with stilbestrol, showed a positive response. Six groups: three treated with estrogen extracts prepared from the milk of cows treated with stilbestrol, and three treated with estrogen extracts prepared from the milk of non-treated cows, showed no response to the treatment. One group which was treated with an estrogen extract prepared from the milk of a normal cow, to which a known quantity of stilbestrol was added, showed a positive response.